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13. ABSTRACT (Maximum 200 Words)

A proportion of breast cancers have genetic alterations at 17q11.2-q12 (HER-2/neu), 20q13.2 (ZNF217 gene) and 17p13.1 (p53). We have documented global genetic instability within malignant breast cancer cell lines (MCF-7; MDA-MB-468 and CRL-2336) by coupling the DNA-damage Comet assay to fluorescence in situ hybridization (Comet-FISH). Gene-specific instability at p53 and HER-2 loci was also observed. Following genotoxic insult (IR and H_2O_2), we found that p53 locus was capable of rapid and preferential repair when compared to the HER-2 locus. Comet-FISH is a rapid and easy assay which is amenable tor the study of gene-specific instability or DNA repair in tissues relating to cancer risk or progression.

14. SUBJECT TERMS

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II. INTRODUCTION

More than one in eight women will be diagnosed with breast cancer in their lifetime. When compared to the general population, the first degree female relatives of breast cancer patients have a two- to threefold increase in breast cancer risk(1). Few biomarker assays are available to identify individuals at risk for breast cancer, given the heterogeneity in shared genetic and/or micro-environmental factors(2-4). Genetic instability is a common finding during the process of tumorigenesis and the presence of non-random chromosome aberrations in breast cancer cells may reflect an underlying predisposition for chromosomal instability at specific gene loci(5). A number of genes have been implicated in familial and sporadic breast cancer carcinogenesis including the estrogen and progesterone receptors, retinoic acid receptors, epidermal growth factor receptor family members (including HER-2/neu), BRCA1, BRCA2, ATM and p53(1). Altered expression of the above genes can be associated with an increased breast cancer risk and altered rates of breast cancer progression(1, 6, 7).

Chromosomal instability within breast cancer cells may arise as a consequence of discordant cell cycle checkpoint control and the repair of specific genetic loci following DNA damage(2, 8). The BRCA1 and BRCA2 genes are prototypes for the association between chromosomal instability, decreased fidelity of DNA repair and an increased risk for breast cancer (7). Mutations in BRCA1 and BRCA2 can prevent appropriate homologous recombination through altered binding to the FANCD2 and RAD51 proteins and inhibit the S or G2 cell cycle checkpoints following DNA damage(6). Other common genetic alterations associated with breast cancers include amplifications of HER-2/neu or ZNF217 (located on 17q11.2-q12 and 20q13.2, respectively) and deletions or mutation in p53 (located on 17p13.1). The HER-2/neu oncogene

encodes a 185 kD trans-membrane tyrosine kinase receptor with extensive homology to the epidermal growth factor receptor. Amplification of HER-2/neu is associated with increased cell proliferation, tumor grade and chromosomal-aneuploidy in association with an aggressive clinical course(9). Breast tumors which express mutations in the p53 gene acquire similar aggressive characteristics, presumably due to altered G1 and G2 cell cycle checkpoint control and decreased DNA repair (10, 11). The oncogene ZNF217 encodes a member of the *Kruppel* family of transcription factors. This gene is amplified early in breast cancer carcinogenesis in one fifth of breast tumors and is associated with poor prognosis (12).

In this study we hypothesized that genetic instability at specific gene loci, such as p53 and HER-2/neu, might be a key characteristic of breast cancer. Indeed, using a combined Comet-FISH protocol, we observed increased instability at both these loci in breast cancer cells. Our data suggests that the ease and rapidity of the Comet-FISH assay could make it useful in the clinical setting for assessing the relative stability of specific gene loci associated with either breast cancer risk or progression.

III. BODY OF REPORT

Original AIMS in Statement of Work:

1) To develop and validate the COMET-FISH assay to detect genetic instability at breast cancer specific loci
2) To apply COMET-FISH to show heterogeneity in genetic instability at these loci

We have completed both AIMS as summarized below in the Methods and Results section.

A) MATERIALS AND METHODS-TECHNOLOGY DEVELOPMENT

Cell Lines and Comet Assay

The breast cancer cell lines used in this study were obtained from ATCC (VA; USA) and included: MCF-7 (also denoted HTB22: $BRCA1^{+/+}$ and $p53^{WT/WT}$); MDA-MB468 (also denoted HTB132: $BRCA1^{+/+}$ and $p53^{MT/WT}$); and CRL2336 ($BRCA1^{-/-}$ and $p53^{MT/-}$). The GM1310B (lymphoblastoid) and AG11134 (normal mammary epithelial) cell lines were obtained from Coriell (NJ; USA). All cell lines were cultured as per the supplier's recommendations.

The alkaline Comet assay was performed essentially as previously described (13, 14). Briefly, cells were admixed with 75 ul of 0.5% low melting agarose at 37°C and spread on a 1% agarose pre-coated slide. Slides were then placed in ice-cold lysis buffer (2.5 M NaCl, 100mM EDTA, 10 mM Trizma base, 10% DMSO, 1% Triton-X) and lysed overnight. After lysis, the slides were placed in horizontal electrophoresis tanks filled with electrophoresis buffer (300 mM NaOH / 1mM EDTA; pH 13.0) for 20 minutes, and then subjected to electrophoresis at 25V/300 mA for a further 20 minutes. After electrophoresis, the slides were washed (0.4 M Tris HCl, pH 7.5; three times 5 minutes each) and dried. For the neutral comet assay, initial steps of the protocol were similar save for additional incubation with Proteinase-K for 60 minutes at 37°C. Additionally, after lysis, slides were placed in horizontal electrophoresis tanks filled with

electrophoresis buffer (1x TBE, pH 8.0) and after 20 minutes, electrophoresis was carried out at 25V/30-45mA for 20 minutes. In both Comet assays, slides were air-dried and stained with ethidium bromide (2μg/ml) prior to scoring. The relative amount of fragmented DNA contained within the Comet's tail, compared to the non-fragmented DNA within the Comet head, was determined by fluorescent image analysis (Northern Eclipse software) (14, 15) to determine the parameters: relative % DNA in tail; comet tail length and Olive tail moment. In selected experiments, GM1310B and HTB22 cells were exposed to 2 or 10 Gy (using a ^{137}Cs source at a dose rate of 1.22 Gy/min as described)(12) or 100 uM H_2O_2 for 5 minutes, to induce DNA double-strand (DNA-dsb) and single-strand (DNA-ssb), breaks, respectively. Comets were then characterized at 0, 15 and 60 minutes post-treatment following addition of fresh media.

Comet-FISH

The Comet assay can be combined with fluorescence in situ hybridization (FISH) methodology to investigate the localization of specific gene loci an individual cell. Whether the FISH signal lies within in the comet head or tail indicates whether the sequence of interest lies within, or in the vicinity of, a damaged region of DNA. FISH probes were obtained from Vysis Inc (Spectrum Orange; Illinois; USA) and included HER-2 (190kb; 17q11.2-q12); ZNF217 (320 kb; 20q13.2) and p53 (145kB; 17p13.1). A 17-Alpha centromeric probe (Spectrum Green) was used as a reference. Exposure to high alkali during the alkaline Comet assay denatures DNA allowing for direct FISH as per the manufacturer's recommendations and previously described(15). FISH probes were denatured at 80°C and applied on to the dehydrated and dried slide and allowed to hybridise overnight in a humidified atmosphere at 37°C. This was followed by gentle washes in 2x SSC at 37°C for 10 minutes before drying at room temperature. The

slides were counter-stained with Sybr® Green and scored immediately using a Nikon Fluorescent microscope fitted with appropriate filters. For neutral Comet-FISH, the DNA was first chemically denatured (0.03M NaOH for 2 minutes at room temperature) prior to probe hybridization.

Initial FISH of cellular cystospins (400 cells per cell line) determined that AG1134, GM1310B, MCF-7 and MDA-MB468 cells had bi-allelic p53 gene signals, whereas CRL-2336 was mono-allelic for p53 (data not shown). AG1134, GM1310B and MCF-7 cells had bi-allelic HER-2 signals. Only 20% cells within the MDA-MB468 and CRL-2336 cell lines had bi-allelic HER-2 signals, the remaining cells in the population with greater than 2 HER-2 signals. All five cell lines were consistently bi-allelic for ZNF217. Subsequent scoring of Comet-FISH signals was carried out by visualising the position of the fluorescent hybridisation signal within the comet "head and tail" profile. The relative distribution of FISH signals in the Comet head versus the comet tail, were interpreted as *stable,* and *unstable,* genetic loci respectively. If signals were in both locations, these were classified as *labile* genetic foci.

RESULTS (SEE APPENDIX I FOR TABLES 1,2 AND ALL FIGURES PERTAINING TO AIMS 1 AND 2 IN STATEMENT OF WORK)

Initial alkaline and neutral Comet assays assessed baseline DNA migration patterns for endogenous DNA breaks relating to ongoing genetic instability within normal (GM1310B and AG1134) and malignant (MCF-7; MDA-MB468 and CRL2336) cell lines(14, 16, 17). Following alkaline lysis, all the breast cancer cell lines showed evidence of increased endogenous DNA breaks (ie. increased Olive tail moments and tail lengths) in comparison to normal cells (see Table 1). This was also true immediately following 2Gy of ionizing radiation.

These data are consistent with increased alkali-labile sites (i.e. DNA single-strand breaks) *de novo* in the fragmented chromatin that migrated outside the breast cancer cell nuclei. A similar trend was observed in the endogenous level of DNA double-strand breaks using the neutral Comet assay. When combined together, both endogenous DNA-dsbs and induced DNA-dsbs were greater in the three breast cancer cell lines, when compared to normal cells ($p<0.05$; Mann-Whitney test). The increased number of initial DNA-ssb or DNA-dsb breaks amongst the breast cancer cell line panel was not related to differential BRCA1 or p53 status/genotype. These results are consistent with increased genetic instability and ongoing DNA damage within malignant breast cells (18, 19).

We subsequently used Comet-FISH to specifically interrogate the endogenous stability of ZNF217, p53, and HER-2 loci. These data are presented in Table 2 with representative images shown in Figure 1. We optimized probe hybridization conditions following alkaline or neutral lysis with the ZNF217 probe. Hybridization of highly-detectable probe (320kb) occurred within 60-70% of alkaline comets and 80-90 % of neutral comets. For the ZNF217 locus, we did not detect differences in hybridization efficiency between normal and cancer cell lines (data not shown). Additionally, less than 5% of ZNF217-FISH signals were observed to be in the tail of the comet in any of the five cell lines, consistent with chromosomal stability at this locus (data not shown). HER-2 or p53 FISH signals were observed in the comet head in greater than 93% of cells in AG1134 or GM1310B cells, consistent with genetic stability at both loci in normal cells. In MCF-7 cells however, only 60 to 73 percent of cells had stable HER-2 and p53 loci. This abnormal pattern increased in the MDA-MB468 and CRL2336 cell lines, in which only 17 to 23 percent of cells had stable HER-2 loci and 43 to 50 percent of cells had stable p53 loci,

respectively. These data are consistent with increased genetic instability at HER-2 and p53 within breast cancer cells and that increased instability can be associated in cells which express a mutant p53 protein and an abrogated G1 checkpoint.

In another series of Comet-FISH experiments, we assessed the location of p53 and HER-2 signals over time following IR or H_2O_2-treatments, as a measure of repair of the allele-containing chromatin domain (see representative images in Figure 2). FISH signals were scattered within the tail of the comet in the majority of AG1134 and MDA-MB468 cells immediately following irradiation or H_2O_2 exposure. Cells were then incubated for 15 or 60 minutes (allowing for potential repair of DNA-ssbs and DNA-dsbs within 1 hour following treatment; a time when maximal repair kinetics occur). At 0 and 15 minutes following either treatment, multiple p53 and HER-2 signals were observed in AG1134 and MDA-MB468 cells. After 60 minutes, both genetic loci were fully repaired in normal cells with no evidence of signal scatter in the comet tail. At the same time-point, the scattered p53 signal resolved to solely 2 detectable signals in the tail of the MDA-MB468 cells, whereas the scattering of the HER-2 signals persisted (cf. Figure 2A and 2B). These data suggest that despite both loci being located on chromosome 17; there is preferential repair of the p53 locus (or it's associated chromatin domain) in both normal and malignant cells.

Further raw background data relating to c-Myc, Cyclin D, ZNF217 and RB loci can be found in Tables 3 to 15 in Appendix II and essentially supports the results of HER-2 and p53 testing above.

IV. Key Research Accomplishments

The Comet assay has been extensively used for toxicological, mutational and DNA repair studies relating to cancer therapy response, carcinogenesis and more recently, genetic stability (reviewed in (17)). By combining the Comet assay with FISH, it is possible to determine gene-specific repair or endogenous genetic instability at specific gene loci. Utilizing this technology, we now show that gene-specific instability occurs at HER-2 and p53 loci in breast cancer cell lines. The two aneuploid and poorly-differentiated cell lines expressing mutant p53, MDA-MB468 and CRL2336, had increased instability at both loci in comparison with the more differentiated and wild type p53-expressing, MCF-7 cells. This data is consistent with clinical data in which breast cancer that express mutant p53 proteins have increased chromosomal instability, resistance to cancer therapy and an increased propensity for distant metastases(1, 11). Importantly, the fact that p53 and HER-2 sequences are specifically released to the tail region within fragmented DNA following DNA damage, suggests that the chromatin structures that underlie these gene domains are particularly vulnerable and may contribute to breast tumor progression in the setting of genetic instability and clonal selection.

Our data in which p53, but not HER-2, sequences are preferentially repaired following DNA damage in breast cancer cell lines, is consistent with a similar study in which the p53 gene was preferentially repaired in irradiated bladder cancer cell lines in comparison to total genomic DNA (17). Why this is the case is currently unclear, but may relate to preferential repair in certain chromosomal domains or during transcription-coupled repair of actively transcribed sequences involved in DNA damage responses following irradiation (6, 17). In the setting of genetic instability, preferential repair and transcription of genetic loci that that are mutated could

lead to increased expression of proteins with altered function and drive a mutator phenotype (5). Further data utilizing isogenic cell lines for specific DNA repair genes of interest would be of interest to follow up this hypothesis.

V. Reportable Outcomes

- A MANUSCRIPT HAS BEEN SUBMITTED TO CANCER EPIDEMIOLOGY, BIOMARKERS AND PREVENTION FEBRUARY 2004 BY KUMARAVEL AND BRISTOW ENTITLED; "DETECTION OF GENETIC INSTABILITY AT HER-2 AND p53 LOCI IN BREAST CANCER CELLS USING COMET-FISH"

- THIS AWARD LED TO THE APPOINTMENT OF DR. KUMARAVEL AS HEAD OF GENETIC AND MOLECULAR TOXICOLOGY, COVANCE LABS LTD., HARROGATE, UK 4G31B4

- THE COMET-FISH ASSAY HAS BEEN VALIDATED USING A SINGLE CELL DIAGNOSTIC METHOD
- THE NEW ASSAY HAS POTENTIAL USES IN CANCER SCREENING AND EPIDEMIOLOGY

VI. Conclusions

The Comet-FISH method has enormous potential in the cancer clinic setting given its ease and rapid generation of multiple and complementary data. The assay can be expanded to the use of multiple simultaneous genetic probes to determine complex relationships between re-arranged geentic loci following DNA damage. Furthermore, the use of Comet-FISH following expsuore to specific DNA-damaging agents and their associated lesions (i.e. creation of DNA breaks, DNA base damage or DNA cross-links) may reveal novel information about gene-specific repair in the context of specific DNA repair pathways(2). From a clinical standpoint, the Comet-FISH assay can be used for any pateint-derived tissue whereby a single-cell suspension can be achieved including peripheral blood lymphocyte, fine needle aspirates or core needle biopsies, brocholavage or urine. For example, given our ability to use Comet-FISH in GM1310B lymphoblasts, the assay could also be used to characterize breast cancer risk by determining the relative instability or DNA damage responses in fibroblasts or peripheral lymphocytes derived from women with strong cancer family histories (3, 4, 20). This may prove to be complementary to BRCA1/BRCA2 mutational analyses as a functional assay for genetic instability(4, 6, 7). Importantly, the assay can also be used as an endpoint to track the efficacy of chemoprevention protocols within serial tissue samples during intervention studies (1, 2).

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VII. Appendices I and II

APPENDIX I: MANUSCRIPT SUBMITTED TO CANCER EPIDEMIOLOGY, BIOMARKERS AND PREVENTION FEBRUARY 2004 BY KUMARAVEL AND BRISTOW ENTITLED; "DETECTION OF GENETIC INSTABILITY AT HER-2 AND p53 LOCI IN BREAST CANCER CELLS USING COMET-FISH"

APPENDIX II: Supplementary data pertaining to c-Myc, RB, ZNF217 and cycin D genes in Tables 3 to 15.

02/04 SHORT COMMUNICATION: CANCER EPIDEM. BIOMARK.PREVENT

DETECTION OF GENETIC INSTABILITY AT HER-2 AND p53 LOCI IN BREAST CANCER CELLS USING COMET-FISH

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Running Title: *Genetic Instability at HER-2 and p53 in Breast Cancer*

Key Words: COMET assay, fluorescent in situ hybridization (FISH), breast cancer, p53, HER-2/neu, genetic instability

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ABSTRACT

A proportion of breast cancers have genetic alterations at 17q11.2-q12 (HER-2/neu), 20q13.2 (ZNF217 gene) and 17p13.1 (p53). We have documented global genetic instability within malignant breast cancer cell lines (MCF-7; MDA-MB-468 and CRL-2336) by coupling the DNA-damage Comet assay to fluorescence in situ hybridization (Comet-FISH). Gene-specific instability at p53 and HER-2 loci was also observed. Following genotoxic insult (IR and H_2O_2), we found that p53 locus was capable of rapid and preferential repair when compared to the HER-2 locus. Comet-FISH is a rapid and easy assay which is amenable tor the study of gene-specific instability or DNA repair in tissues relating to cancer risk or progression.

INTRODUCTION

More than one in eight women will be diagnosed with breast cancer in their lifetime. When compared to the general population, the first degree female relatives of breast cancer patients have a two- to threefold increase in breast cancer risk(1). Few biomarker assays are available to identify individuals at risk for breast cancer, given the heterogeneity in shared genetic and/or micro-environmental factors(2-4). Genetic instability is a common finding during the process of tumorigenesis and the presence of non-random chromosome aberrations in breast cancer cells may reflect an underlying predisposition for chromosomal instability at specific gene loci(5). A number of genes have been implicated in familial and sporadic breast cancer carcinogenesis including the estrogen and progesterone receptors, retinoic acid receptors, epidermal growth factor receptor family members (including HER-2/neu), BRCA1, BRCA2, ATM and p53(1). Altered expression of the above genes can be associated with an increased breast cancer risk and altered rates of breast cancer progression(1, 6, 7).

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respectively) and deletions or mutation in p53 (located on 17p13.1). The HER-2/neu oncogene encodes a 185 kD trans-membrane tyrosine kinase receptor with extensive homology to the epidermal growth factor receptor. Amplification of HER-2/neu is associated with increased cell proliferation, tumor grade and chromosomal-aneuploidy in association with an aggressive clinical course(9). Breast tumors which express mutations in the p53 gene acquire similar aggressive characteristics, presumably due to altered G1 and G2 cell cycle checkpoint control and decreased DNA repair (10, 11). The oncogene ZNF217 encodes a member of the *Kruppel* family of transcription factors. This gene is amplified early in breast cancer carcinogenesis in one fifth of breast tumors and is associated with poor prognosis (12).

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MATERIALS AND METHODS

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The alkaline Comet assay was performed essentially as previously described (13, 14). Briefly, cells were admixed with 75 ul of 0.5% low melting agarose at 37°C and spread on a 1% agarose pre-coated slide. Slides were then placed in ice-cold lysis buffer (2.5 M NaCl, 100mM EDTA, 10 mM Trizma base, 10% DMSO, 1% Triton-X) and lysed overnight. After lysis, the slides were placed in horizontal electrophoresis tanks filled with electrophoresis buffer (300 mM NaOH / 1mM EDTA; pH 13.0) for 20 minutes, and then subjected to electrophoresis at 25V/300 mA for a further 20 minutes. After electrophoresis, the slides were washed (0.4 M Tris HCl, pH 7.5; three times 5 minutes each) and dried. For the neutral comet assay, initial steps of the protocol were similar save for additional incubation with Proteinase-K for 60 minutes at 37°C. Additionally, after lysis, slides were placed in horizontal electrophoresis tanks filled with electrophoresis buffer (1x TBE, pH 8.0) and after 20 minutes, electrophoresis was carried out at 25V/30-45mA for 20 minutes. In both Comet assays, slides were air-dried and stained with ethidium bromide (2μg/ml) prior to scoring. The relative amount of fragmented DNA contained within the Comet's tail, compared to the non-fragmented DNA within the Comet head, was determined by fluorescent image analysis (Northern Eclipse software) (14, 15) to determine the parameters: relative % DNA in tail; comet tail length and Olive tail moment. In selected experiments, GM1310B and HTB22 cells were exposed to 2 or 10 Gy (using a ^{137}Cs source at a dose rate of 1.22 Gy/min as described)(12) or 100 uM H_2O_2 for 5 minutes, to induce DNA double-strand (DNA-dsb) and single-strand (DNA-ssb), breaks, respectively. Comets were then characterized at 0, 15 and 60 minutes post-treatment following addition of fresh media.

Comet-FISH

The Comet assay can be combined with fluorescence in situ hybridization (FISH) methodology to investigate the localization of specific gene loci an individual cell. Whether the FISH signal lies within in the comet head or tail indicates whether the sequence of interest lies within, or in the vicinity of, a damaged region of DNA. FISH probes were obtained from Vysis Inc (Spectrum Orange; Illinois; USA) and included HER-2 (190kb; 17q11.2-q12); ZNF217 (320 kb; 20q13.2) and p53 (145kB; 17p13.1). A 17-Alpha centromeric probe (Spectrum Green) was used as a reference. Exposure to high alkali during the alkaline Comet assay denatures DNA allowing for direct FISH as per the manufacturer's recommendations and previously described(15). FISH probes were denatured at 80°C and applied on to the dehydrated and dried slide and allowed to hybridise overnight in a humidified atmosphere at 37°C. This was followed by gentle washes in 2x SSC at 37°C for 10 minutes before drying at room temperature. The slides were counter-stained with Sybr® Green and scored immediately using a Nikon Fluorescent microscope fitted with appropriate filters. For neutral Comet-FISH, the DNA was first chemically denatured (0.03M NaOH for 2 minutes at room temperature) prior to probe hybridization.

Initial FISH of cellular cystospins (400 cells per cell line) determined that AG1134, GM1310B, MCF-7 and MDA-MB468 cells had bi-allelic p53 gene signals, whereas CRL-2336 was mono-allelic for p53 (data not shown). AG1134, GM1310B and MCF-7 cells had bi-allelic HER-2 signals. Only 20% cells within the MDA-MB468 and CRL-2336 cell lines had bi-allelic HER-2 signals, the remaining cells in the population with greater than 2 HER-2 signals. All five

cell lines were consistently bi-allelic for ZNF217. Subsequent scoring of Comet-FISH signals was carried out by visualising the position of the fluorescent hybridisation signal within the comet "head and tail" profile. The relative distribution of FISH signals in the Comet head versus the comet tail, were interpreted as *stable,* and *unstable,* genetic loci respectively. If signals were in both locations, these were classified as *labile* genetic foci.

RESULTS

Initial alkaline and neutral Comet assays assessed baseline DNA migration patterns for endogenous DNA breaks relating to ongoing genetic instability within normal (GM1310B and AG1134) and malignant (MCF-7; MDA-MB468 and CRL2336) cell lines(14, 16, 17). Following alkaline lysis, all the breast cancer cell lines showed evidence of increased endogenous DNA breaks (ie. increased Olive tail moments and tail lengths) in comparison to normal cells (see Table 1). This was also true immediately following 2Gy of ionizing radiation. These data are consistent with increased alkali-labile sites (i.e. DNA single-strand breaks) *de novo* in the fragmented chromatin that migrated outside the breast cancer cell nuclei. A similar trend was observed in the endogenous level of DNA double-strand breaks using the neutral Comet assay. When combined together, both endogenous DNA-dsbs and induced DNA-dsbs were greater in the three breast cancer cell lines, when compared to normal cells ($p<0.05$; Mann-Whitney test). The increased number of initial DNA-ssb or DNA-dsb breaks amongst the breast cancer cell line panel was not related to differential BRCA1 or p53 status/genotype. These results are consistent with increased genetic instability and ongoing DNA damage within malignant breast cells (18, 19).

We subsequently used Comet-FISH to specifically interrogate the endogenous stability of ZNF217, p53, and HER-2 loci. These data are presented in Table 2 with representative images shown in Figure 1. We optimized probe hybridization conditions following alkaline or neutral lysis with the ZNF217 probe. Hybridization of highly-detectable probe (320kb) occurred within 60-70% of alkaline comets and 80-90 % of neutral comets. For the ZNF217 locus, we did not detect differences in hybridization efficiency between normal and cancer cell lines (data not shown). Additionally, less than 5% of ZNF217-FISH signals were observed to be in the tail of the comet in any of the five cell lines, consistent with chromosomal stability at this locus (data not shown). HER-2 or p53 FISH signals were observed in the comet head in greater than 93% of cells in AG1134 or GM1310B cells, consistent with genetic stability at both loci in normal cells. In MCF-7 cells however, only 60 to 73 percent of cells had stable HER-2 and p53 loci. This abnormal pattern increased in the MDA-MB468 and CRL2336 cell lines, in which only 17 to 23 percent of cells had stable HER-2 loci and 43 to 50 percent of cells had stable p53 loci, respectively. These data are consistent with increased genetic instability at HER-2 and p53 within breast cancer cells and that increased instability can be associated in cells which express a mutant p53 protein and an abrogated G1 checkpoint.

In another series of Comet-FISH experiments, we assessed the location of p53 and HER-2 signals over time following IR or H_2O_2-treatments, as a measure of repair of the allele-containing chromatin domain (see representative images in Figure 2). FISH signals were scattered within the tail of the comet in the majority of AG1134 and MDA-MB468 cells immediately following irradiation or H_2O_2 exposure. Cells were then incubated for 15 or 60 minutes (allowing for potential repair of DNA-ssbs and DNA-dsbs within 1 hour following

treatment; a time when maximal repair kinetics occur). At 0 and 15 minutes following either treatment, multiple p53 and HER-2 signals were observed in AG1134 and MDA-MB468 cells. After 60 minutes, both genetic loci were fully repaired in normal cells with no evidence of signal scatter in the comet tail. At the same time-point, the scattered p53 signal resolved to solely 2 detectable signals in the tail of the MDA-MB468 cells, whereas the scattering of the HER-2 signals persisted (cf. Figure 2A and 2B). These data suggest that despite both loci being located on chromosome 17; there is preferential repair of the p53 locus (or it's associated chromatin domain) in both normal and malignant cells.

DISCUSSION

The Comet assay has been extensively used for toxicological, mutational and DNA repair studies relating to cancer therapy response, carcinogenesis and more recently, genetic stability (reviewed in (17)). By combining the Comet assay with FISH, it is possible to determine gene-specific repair or endogenous genetic instability at specific gene loci. Utilizing this technology, we now show that gene-specific instability occurs at HER-2 and p53 loci in breast cancer cell lines. The two aneuploid and poorly-differentiated cell lines expressing mutant p53, MDA-MB468 and CRL2336, had increased instability at both loci in comparison with the more differentiated and wild type p53-expressing, MCF-7 cells. This data is consistent with clinical data in which breast cancer that express mutant p53 proteins have increased chromosomal instability, resistance to cancer therapy and an increased propensity for distant metastases(1, 11). Importantly, the fact that p53 and HER-2 sequences are specifically released to the tail region within fragmented DNA following DNA damage, suggests that the chromatin structures that

underlie these gene domains are particularly vulnerable and may contribute to breast tumor progression in the setting of genetic instability and clonal selection.

Our data in which p53, but not HER-2, sequences are preferentially repaired following DNA damage in breast cancer cell lines, is consistent with a similar study in which the p53 gene was preferentially repaired in irradiated bladder cancer cell lines in comparison to total genomic DNA (17). Why this is the case is currently unclear, but may relate to preferential repair in certain chromosomal domains or during transcription-coupled repair of actively transcribed sequences involved in DNA damage responses following irradiation (6, 17). In the setting of genetic instability, preferential repair and transcription of genetic loci that that are mutated could lead to increased expression of proteins with altered function and drive a mutator phenotype (5). Further data utilizing isogenic cell lines for specific DNA repair genes of interest would be of interest to follow up this hypothesis.

The Comet-FISH method has enormous potential in the cancer clinic setting given its ease and rapid generation of multiple and complementary data. The assay can be expanded to the use of multiple simultaneous genetic probes to determine complex relationships between re-arranged geentic loci following DNA damage. Furthermore, the use of Comet-FISH following expsuore to specific DNA-damaging agents and their associated lesions (i.e. creation of DNA breaks, DNA base damage or DNA cross-links) may reveal novel information about gene-specific repair in the context of specific DNA repair pathways(2). From a clinical standpoint, the Comet-FISH assay can be used for any pateint-derived tissue whereby a single-cell suspension can be achieved including peripheral blood lymphocyte, fine needle aspirates or core needle

biopsies, brocholavage or urine. For example, given our ability to use Comet-FISH in GM1310B lymphoblasts, the assay could also be used to characterize breast cancer risk by determining the relative instability or DNA damage responses in fibroblasts or peripheral lymphocytes derived from women with strong cancer family histories (3, 4, 20). This may prove to be complementary to BRCA1/BRCA2 mutational analyses as a functional assay for genetic instability(4, 6, 7). Importantly, the assay can also be used as an endpoint to track the efficacy of chemoprevention protocols within serial tissue samples during intervention studies (1, 2).

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TABLE 1: INITIAL BASELINE AND IR-INDUCED DNA DAMAGE IN NORMAL AND MALIGNANT BREAST CANCER CELLS

(i) Alkaline Comets

Cell lines	Base line DNA damage			DNA damage after pre-imposed 2 Gy radiation		
	% DNA	Tail Length	Olive Tail Moment	% DNA	Tail Length	Olive Tail Moment
GM1310B	11.9 ± 2.8	21.3 ± 5.2	2.1 ± 0.5	33.1 ± 7.3	37.1 ± 4.5	8.1± 1.1
AG11134	12.5 ± 3.1	24.1 ± 7.2	1.9 ± 0.5	35.6 ± 6.7	39.6 ± 6.1	7.1 ± 1.4
HTB22	18.3 ± 3.1	29.5 ± 5.3	3.0 ± 0.8	45.1 ± 8.2	48.1 ± 4.8	10.3 ± 2.1
HTB132	16.9 ± 5.2	28.8 ± 4.1	3.1 ± 1.0	42.7 ± 3.2	49.6 ± 6.2	9.8 ± 1.2
CRL2336	19.2 ± 5.7	30.1 ± 5.2	3.0 ± 0.9	48.1 ± 6.6	51.2 ± 7.6	10.3 ± 2.9

(ii) Neutral Comets

Cell lines	Base line DNA damage			DNA damage after pre-imposed 10 Gy radiation		
	% DNA	Tail Length	Olive Tail Moment	% DNA	Tail Length	Olive Tail Moment
GM1310B	10.1 ± 2.3	20.9 ± 1.4	0.8 ± 0.1	30.7 ± 5.6	36.2 ± 4.2	10.1 ± 2.7
AG11134	14.4 ± 2.1	19.2 ± 1.7	0.9 ± 0.2	28.8 ± 6.3	38.9 ± 5.1	10.4 ± 2.9
HTB22	20.2 ± 4.2	31.7 ± 3.1	1.2 ± 0.4	38.1 ± 7.1	45.1 ± 6.1	15.2 ± 3.1
HTB132	19.8 ± 4.8	30.9 ± 4.7	1.8 ± 0.5	42.4 ± 6.4	45.4 ± 5.3	15.5 ± 4.2
CRL2336	21.7 ± 4.9	28.7 ± 5.2	1.5 ± 0.4	40.8 ± 7.8	43.5 ± 6.1	14.6 ± 3.9

All values are (mean of 3 independent experiments) ± (standard error of the mean).

TABLE 2. GENETIC INSTABILITY AT p53 and HER-2 LOCI

(i) HER-2/neu

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	81	1	1	17
AG11134	87	2	0	11
HTB22	56	11	10	23
HTB132	19	20	43	18
CRL2336	14	18	50	18

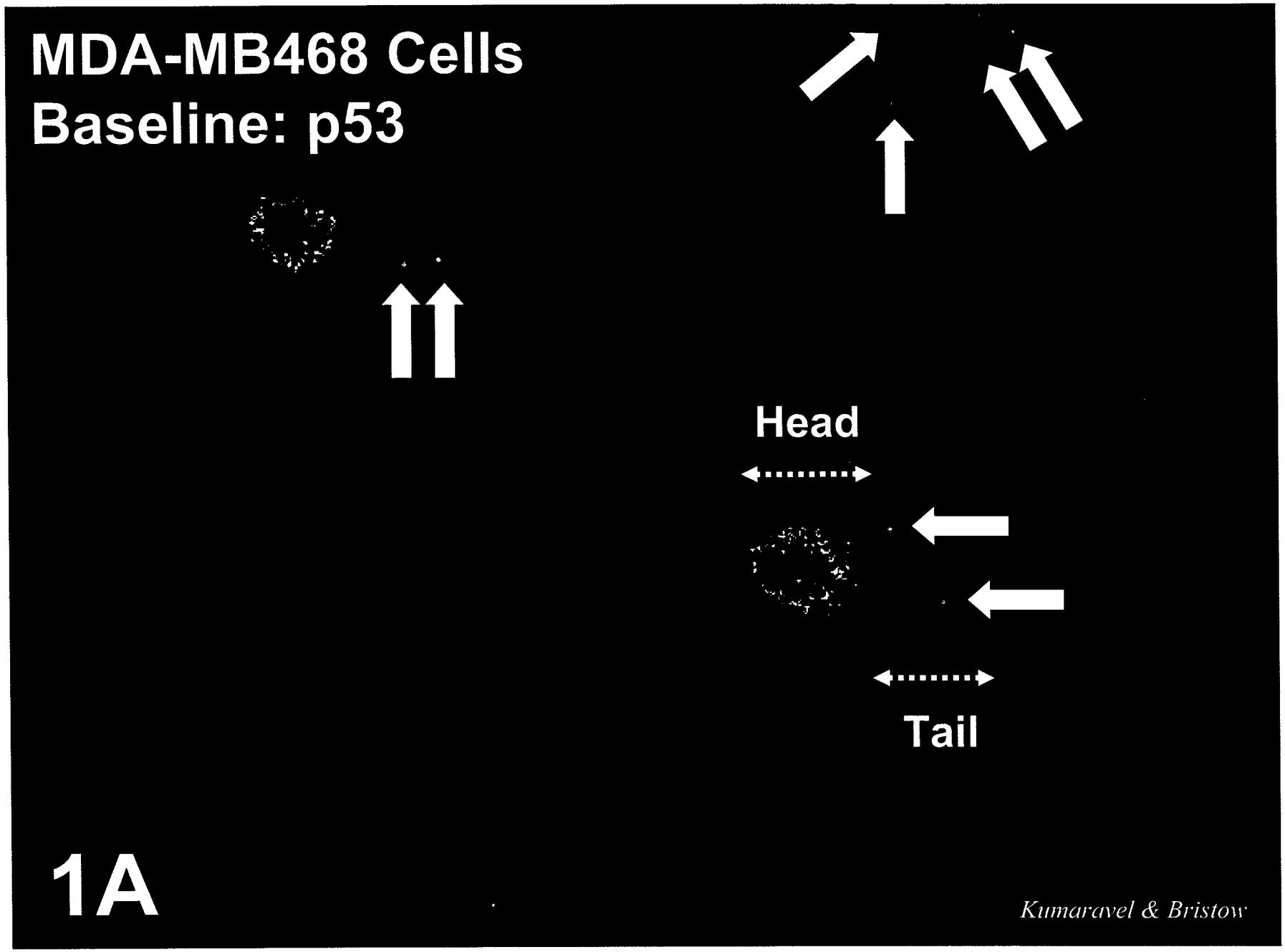
(ii) p53

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	81	4	0	15
AG11134	75	3	2	20
HTB22	51	19	14	16
HTB132	45	16	29	10
CRL2336	37	21	28	14

FIGURE LEGENDS

Figure 1. (A) MDA-MB468 breast cancer cells prior to DNA damage showing baseline p53 FISH signals in the tail of the comets. Also shown is a cell with multiple small baseline p53 signals in the tail. The "head" and "tail" of the comet is shown. (B) AG11134 normal human mammary epithelial cells prior to DNA damage showing baseline p53 FISH signals in the head of the comet. A similar observation was made for the location of HER-2 FISH signals in this cell line. (C) MDA-MB468 breast cancer cells prior to DNA damage showing HER-2 FISH signals in the tail of the comets. Similar cells could be found which had p53 signals within the comet tail. In each image above, the DNA is counterstained with either propidium iodide (red) or DAPI (blue)

Figure 2. (A) MDA-MB468 breast cancer cells at 15 minutes following exposure to 100 μM H_2O_2. Numerous p53 signals are scattered throughout the tail of the comet. (B) MDA-MB468 breast cancer cells at 60 minutes following exposure to 100 μM H_2O_2. Scattering of the p53 FISH signals have resolved, leaving 2 discrete p53 signals in the tail of the comet.


MDA-MB468 Cells
Baseline: p53
Head
Tail
1A
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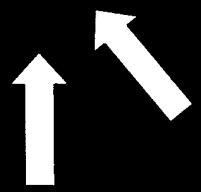
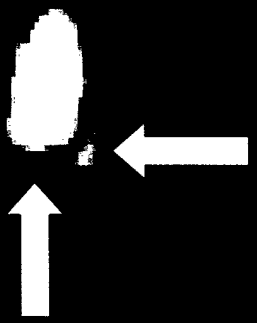
AG1134 Cells
Baseline: p53





1B

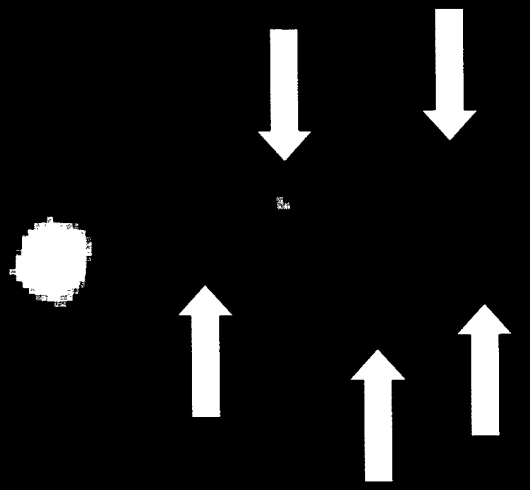
Kumaravel & Bristow

MDA-MD-468 Cells
Baseline: HER-2

1C

Kumaravel & Bristow

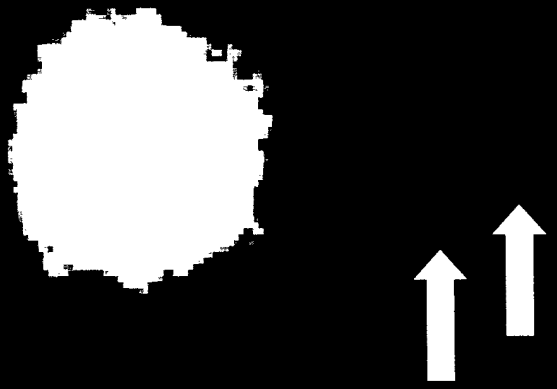
MDA-MB468 Cells
H2O2 (15 min): p53



2A

Kumaravel & Bristow

MDA-MB-468Cells
H2O2 (60min): p53



2B

Kumaravel & Bristow

BASELINE FISH OBSERVATIONS (TABLES 3, 4, AND 5)

Table 3. List of probes used in this study.

Probes	Probe Length	Gene Loci	Reference Probes
HER2 (Spectrum Orange)	~190 kb	17q11.2-q12	17 Alpha centromeric probe (Spectrum Green)
ZNF217 (Spectrum Orange)	~320 kb	20q13.2	-
Cyclin D (Spectrum Orange)	~300 kb	11q13	-
p53 (Spectrum Orange)	~145 kb	17p13.1	-
c-myc (Spectrum Orange)	~120 kb	8q24.12-q24.13	-
Rb (Spectrum Orange)	~220 kb	13q14.3	-

All probes from Vysis Inc.

Table 4. Baseline FISH observations using different FISH probes.

Cell lines	Percentage of cells with 2 signals				
	ZNF217	Cyclin D	p53	c-myc	Rb
GM1310B	88	87	90	92	84
AG11134	87	89	86	89	87
HTB22	90*	89	85	89*	54#
HTB132	85*	87	92	91*	28#
CRL2336	83*	75	84	81*	49#

* no evidence suggestive of amplification; # deletion of Rb gene seen in these cell lines
Cells were cytospin, fixed with Carnoy's fixative, and FISH performed as per the probe manufacturer's recommendation. a total of 400 cells were scored from each sample.

Table 5. Baseline FISH observations using the HER-2 probes.

Cell lines	HER-2
GM1310B	92% with 2 signals, good hybridisation efficiency
AG11134	95% with 2 signals, good hybridisation efiiciency
HTB22	81% with 2 signals; 8% with more than 2 signals; good hybridisation efficiency
HTB132	26% with 2 signals; 60 % more than 2 signals; good hybridisation efficiency
CRL2336	21% with 2 signals; 70% with more than 2 signals; good hybridisation efficiency

Cells were cytospin, fixed with Carnoy's fixative, and FISH performed as per the probe manufacturer's recommendation. a total of 400 cells were scored from each sample.

VALIDATION OF COMET-FISH METHOD (TABLES 6, 7, 8 AND 9)

ZNF217 probe was selected for the validation studies because of its large size (~320 kb).

Table 6. Classification system for Comet-FISH

Distribution of FISH signals in the comet	Interpretation
FISH signals in the Head	Stable genetic loci
Signals both in head and tail	labile genetic loci
FISH signals in the Tail	Unstable genetic loci

Table 7. Fluorescence in situ hybridisation with ZNF217 probe following alkaline Comet assay

	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	51	14	2	33
AG11134	45	7	3	45
HTB22	32	18	7	43
HTB132	37	19	5	39
CRL2336	40	9	9	42

The slides were hybridised with FISH probes following Alkaline Comet assay. Alkaline treatment at pH>13 would denature the DNA and help in probe hybridisation

Table 8. Florescence in situ hybridisation with ZNF217 probe on Neutral Comet assay slides denatured with 70% formamide at 72°C.

	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	63	11	5	21
AG11134	61	6	7	26
HTB22	59	2	10	29
HTB132	61	5	9	25
CRL2336	55	2	15	28

The slides were hybridised with FISH probes following Alkaline Comet assay. The alkaline treatment at pH>13 would denature the DNA and help in probe hybridisation

Table 9. Florescence in situ hybridisation with ZNF217 probe on Neutral Comet assay slides denatured with 0.03M NaOH, °C for 2.5 minutes, neutralised in 0.4M Tris (5 min) and TBE (2 min).

	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	73	9	5	13
AG11134	75	12	5	8
HTB22	71	9	5	15
HTB132	76	7	8	9
CRL2336	73	10	6	11

GENETIC INSTABILITY IN BREAST CANCER CELL LINES (TABLES 10-14)

Table 10. Genetic Instability at HER-2 loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	81	1	1	17
AG11134	87	2	0	11
HTB22	56	11	10	23
HTB132	19	20	43	18
CRL2336	14	18	50	18

Table 11. Genetic Instability at p53 loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	81	4	0	15
AG11134	75	3	2	20
HTB22	51	19	14	16
HTB132	45	16	29	10
CRL2336	37	21	28	14

Table 12. Genetic Instability at cyclin-D loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	79	1	3	17
AG11134	85	3	0	12
HTB22	81	1	5	13
HTB132	77	5	2	16
CRL2336	71	2	1	26

Table 13. Genetic Instability at c-myc loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	84	3	5	8
AG11134	78	7	3	12
HTB22	72	11	7	10
HTB132	76	9	4	11
CRL2336	71	2	7	20

Table 14. Genetic Instability at RB loci in Breast cancer cell lines.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
GM1310B	75	5	12	8
AG11134	66	5	17	12
HTB22	13	12	14	61
HTB132	34	11	21	34
CRL2336	16	7	17	60

RESULTS OF GENETIC INSTABILITY IN APPARENTLY NORMAL INDIVIDUALS (TABLE 15)

Table 15. Genetic Instability in apparently normal individuals.

Cell lines	Percent cells with both signals in the head	Percent cells with one signal in the tail and one in head	Percent cells with both signals in the tail	Percent cells with no FISH signals
HER-2				
A	86	3	1	10
B	81	2	0	17
P53				
A	89	5	1	5
B	83	7	1	9
ZNF217				
A	88	5	2	5
B	88	5	0	7
c-myc				
A	79	8	2	11
B	80	4	1	15
Cyclin-D				
A	83	8	0	9
B	71	11	1	17
Rb				
A	92	0	0	8
B	93	0	0	7